Identification of peptide L, a novel neuropeptide that regulates the expression of L-type voltage-gated calcium channels in photoreceptors

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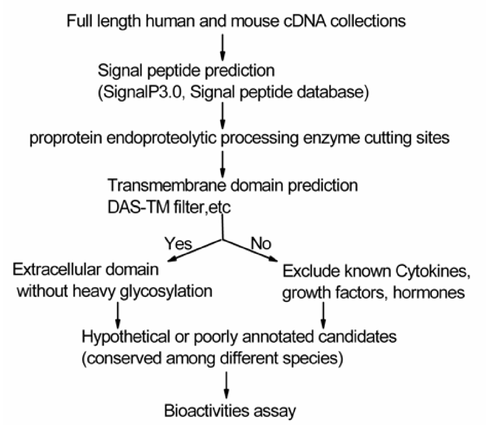
Introduction:

- Neuropeptides act like peptidic hormones or neurotransmitters and play diverse roles in regulating neural functions (1).
- Computational bioinformatics has become a powerful tool to discover novel bioactive peptides (2).
- The L-type voltage-gated calcium channels (L-VGCCs) mediate a voltage-dependent, depolarization-induced Ca2+ influx and regulate diverse biological processes such as contraction, secretion, differentiation, synaptic plasticity, and gene expression (3).
- The L-VGCCs are essential in gating pro-longed neurotransmitter release in retinal photoreceptors (4).

Methods:

Cloning of mouse peptide L full length cDNA
Cell culture and transfection
High-performance liquid chromatography (HPLC) and mass spectrometry (MS)
In situ hybridization
Immunoblotting, quantitative (Q)-PCR, and cAMP enzyme immunoassay
GST fusion protein purification and peptide synthesis
Patch Clamp Electrophysiology

Figure 1. A flow chart illustration of our *in silico* computational screening strategy and procedure.



Human and mouse full length protein databases were subjected to a separation with five steps by computer algorithms including N- terminal signal peptide sequence (secretion marker), propeptide convertase targeting motif, transmembrane domain, potential glycosylation modification, and sequence homology across species. Potential candidates were ranked according to the score assigned from each step.L (5805.6982 m/z). The 49 amino acid sequence of peptide L is listed at the top of this panel.

Figure 2. Sequence alignment between human and mouse propeptide L.

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Protein sequence alignment

Human   --MRLLALAAAALLARAPAPEVCAALNVTVSPGPVVDYLEGENATLLCHVSQKRRKDSLL 58
Mouse   MRLRLLALAAAAVLLG--PAPEVCGALNVTVSPGPVVDYLEGENATLLCHVSQKRRKDSLL 58
          :********.**.  ******.************************************
Human   AVIWFFAHSPDSQEALMVKMTKLRIIQYYGNFSRTANQQRLRLLEERRSVLYRLSVLTLQ 118
Mouse   AVEWFFAPD-QSQEALMVKMTKLRIIQYYGNFSRTANQQRLRLLEERRSVLYRLSVLTLR 117
        ******* . .************::********:*:::*******:**.*********:
Human   PSDQGHYVCRVQEISRHRNKWTAWSNGSSATEMRVISLKASEESSFEKTKETWAFFEDLY 178
Mouse   PTDQGQYVCRVQEISKHRNKWTAWSKGSSATEMRVISLKAGEDSSFEKKKVTWAFFEDLY 177
        *:***:***:*****:**************************.*:*****.* *********
Human   VYAVLVCCVGILSILLFMLVIVWQSVFNKRKSRVRHYLVKCPQNSSGETVTSVTSLAPLQ 238
Mouse   VYAVLVCCVGILSVLLFTLVIAWQSVFHKRKSRVRHYLVKCPQNSSGETVTSVTSLAPLQ 237
        ************:*** ***.*****:**********************************
Human   PKKGKRQKEKPDIPPAVPAKAPIAPTFHKPKLLKPQRKVTLPKIAEENLTYAELELIKPH 298
Mouse   PQKGKRQKKKVDVPPAVPAKAPIATTFHKPKLLKPQRKVALPKITEENLTYAELELIKPH 297
        *:******:* *:***********.**************:****:***************
Human   RAAKGAPTSTVYAQILFEENKL 320
Mouse   RAAKGVPTSTVYAQILFEENQL 319
        *****.**************:*

Signal peptide   Propeptide convertase cutting site
Transmembrane domain   Peptide L   Peptide II
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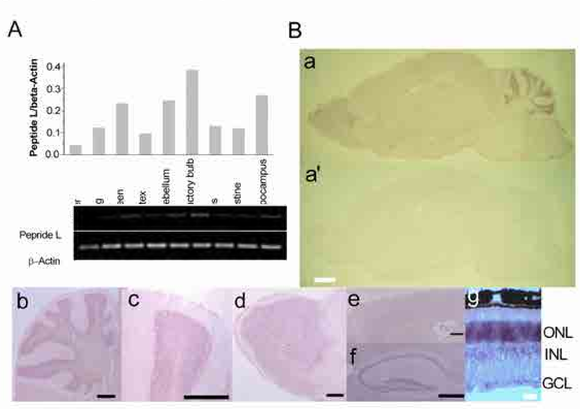
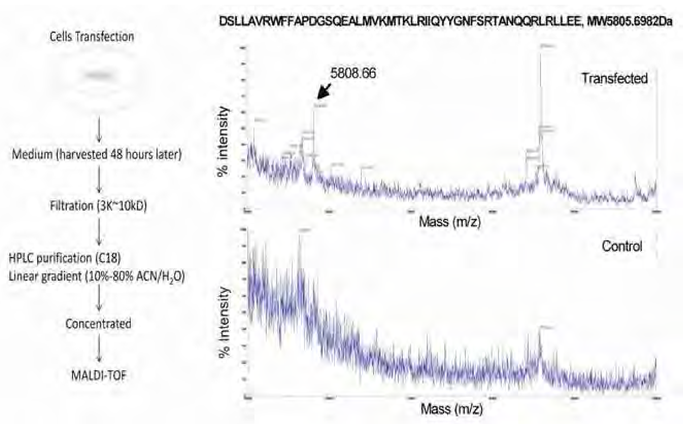
Human and mouse peptide L precursor are over 80% homologous. The sequence contains a signal peptide sequence (green), proprotein convertase cutting motifs (red), the main peptide L coding region (blue), and a transmembrane domain (purple). Peptide II (control for the electrophysiological studies) is highlighted in orange.

Figure 3. Expression of peptide L in mouse tissues.



(A) Gene expression of peptide L in various mouse tissues was determined by RT-PCR. The mRNA of peptide L was detected in the liver, lung, spleen, intestine, eyes, cerebral cortex (cortex), cerebellum, olfactory bulb, and hippocampus, with relatively higher expression levels in the spleen, cerebellum, olfactory bulb, and hippocampus. (B) The mRNA expression of peptide L in the mouse brain and retina was detected by *in situ* hybridization. Adult mouse brain sagittal sections and retina sections were cut at 10 μm thickness and processed for *in situ* hybridization. The sense probe served as the negative control (a'). Propeptide L is strongly expressed in the olfactory bulb (a, d), cerebellum (a, d, c), cerebral cortex (a, e), and hippocampus (a, c). In the mouse retina (g), the expression of peptide L was detected in the outer nuclear layer (ONL, strongest signal), inner nuclear layer (INL), and ganglion cell layer (GCL). The scale bars represent 1.0 mm (a and a'), 0.1 mm (b, c, d, e, f), and 0.02 mm (g).

Figure 4. Verification of peptide L secretion by MALDI-TOF mass spectrometry.



Mouse 661w cells were transfected with the propeptide L gene (mouse E130203B14Rik) expression vector or empty vector (control). Culture media were collected 36-40 hrs after transfection, filtered (3~10kD), and subjected to HPLC. The final elution (linear gradient 10%-80% ACN/H_2O and 0.1% TFA in all solutions) was concentrated by speedvac and analyzed by MALDI-TOF MS. The arrowhead indicates the peak (5808.66 m/z) that corresponds to the predicted molecular weight of peptide L (5805.6982 m/z). The 49 amino acid sequence of peptide L is listed at the top of this panel.

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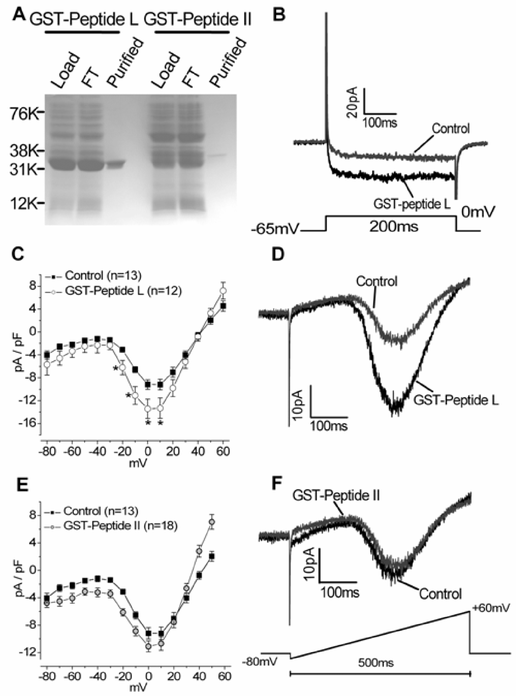
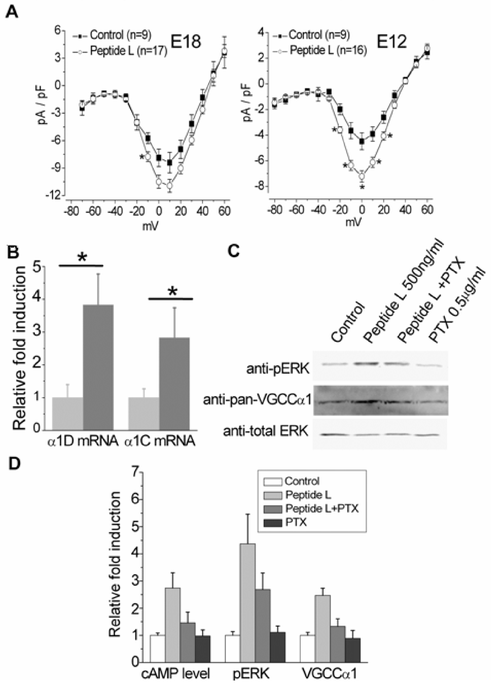
Figure 5. GST-peptide L increased L-VGCC currents in cone photoreceptors.



GST-peptide L and GST-peptide II (control) were expressed and purified from *E. coli* (A). Cultured E18 cone photoreceptors were treated with PBS (control), 800 ng/ml GST-peptide II (control peptide), or 800 ng/ml GST-peptide L for 4 hr prior to recordings. Whole cell patch clamp recordings were performed under both ramp and step commands (B, D). Representative current traces under a ramp command (D, F) or a step command (E) are shown. Average current-voltage (I-V) relationships were obtained from the step command as in current density (pA/pF), and the maximum inward current densities were elicited at 0 - 10mV of the step command (C, E). The average maximal current density obtained from GST-peptide L treated cells (n=12, open circle, C) was significantly larger than the control (n=13; filled square). No significant difference occurred between the control and GST-peptide II treated cells (n=18; gray circle; E). Comparisons between the control and recombinant protein groups were made using Student's *t*-test; *$p < 0.05$.

Figure 6. Synthesized peptide L enhanced the protein expression L-VGCCα1 subunits, cAMP production, and ERK phosphorylation in chicken cone photoreceptors.



Cultured chick photoreceptors (E10+2 and E16+2) were treated with 500 ng/ml synthesized peptide L or control buffer for 4 hrs prior to patch clamp recordings (A). Averaged current-voltage (I-V) relationships were obtained from the step command as in current density (pA/pF), and the maximum inward current densities were elicited at 0-10 mV of the step command. Peptide L increased current density at both E12 and E18 (compared to their respective control groups). E12: control, -4.49 ± 0.66 pA/pF (n=9, dark square); peptide L treated cells, -7.22 ± 0.45 pA/pF (n=16, open square). E18: control, -8.40 ± 1.11 pA/pF (n=9, dark square); peptide L, -10.93 ± 0.70 pA/pF (n=17, open square). The mRNA levels of both L-VGCCα1C and α1D were measured by Q-PCR (B; n=6 for each group). Comparisons between the control and peptide L groups were made using Student's *t*-test; * $p < 0.05$. L-VGCCα1, ERK, and pERK were detected by western blot. The band densities were measured and relative values were calculated as a ratio to total ERK (C). Relative fold difference (n=3) was calculated by comparison to control samples which were set to one (D). Peptide L significantly increased cAMP levels, phosphorylation of ERK, and L-VGCCα1 expression (D, gray bar). However, PTX, a specific GPCR inhibitor, blocked peptide L effects (D, dark gray bar). Values are means±sem.

Conclusion:

- Computational bioinformatics coupled with electrophysiological activities assay can be an effective method to identify bioactive neuropeptides.
- Peptide L up-regulated the expression of L-VGCCs in photoreceptors.
- Peptide L might be important in modulating neurotransmitter release in photoreceptors.

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